bio-protocol Preprint

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Isolation of T-cell subpopulations

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An abbreviated version of this protocol was published in eLIFE in Feb 2020

Primary and secondary anti-viral response captured by the dynamics and phenotype of individual T cell clones

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Detailed protocol

We isolated PBMCs from the blood using standard Ficoll-Paque protocol.
PBMC isolation:

1. Collect blood into EDTA tubes (Heparin may inhibit following RT reaction).
2. Dilute blood 4 times with PBS.
3. Put 6 or 15 mL of room temperature Ficoll into 15 or 50 mL tubes.
4. Gently lay diluted blood on top of Ficoll preventing layer mixing.
5. Centrifuge 30 minutes, 400 g, room temperature on bucket-rotor centrifuge with minimal acceleration/deceleration.
6. Discard the plasma. Collect the white buffy coat of cells to a separate tube.
7. Wash twice with 15-50 mL PBS. Centrifuge 15 minutes, 450 g, room temperature.
8. For each replicate of bulk PBMC TCR repertoire, ~5 mln of cells was lysed with 1 mL Trizol reagent and stored at -80 for several months.

CD4 and CD8 fractions were isolated with CD4/CD8 Positive Selection Dynabeads Kits according to the manufacturer's protocol. CD8 positive isolation was performed first, followed by the CD4 positive isolation from the flowthrough.

1. PBMCs were resuspended in 1 mL Dynabeads buffer (PBS(Ca2+ and Mg2+ free) with 0.1% BSA and 2 mM EDTA, pH 7.4.).
2. Resuspend the Dynabeads® in the vial (vortex >30 sec or tilt and rotate for 5 min).
3. Transfer 25 ul of Dynabeads® to a tube.
4. Add 1 mL of Dynabeads buffer and resuspend.
5. Place the tube in a magnet for 1 min and discard the supernatant.
6. Remove the tube from the magnet and resuspend the washed Dynabeads® in 25 ul.
7. Wash PBMCs with 1 mL cold Dynabeads buffer..
8. Centrifuge at 500 × g for 10 min at 2°C to 8°C. Allow to decelerate slowly.
9. Resuspend cells in 1 mL cold Dynabeads buffer and add them to 25 ul washed Dynabeads.
10. Incubate for 20 min at 2°C to 8°C with gentle tilting and rotation.
11. Place the tube in a magnet for 2 min.
12. While the tube is still in the magnet, carefully remove and put supernatant to 25 ul washed anti-CD4 Dynabeads (for washing see steps 2-6). Repeat exactly the same isolation procedure.
13. Remove the tube from the magnet and add 1 mL Buffer 1, pipet 2–3 times (or vortex 2–3 seconds) and place the tube in a magnet for 2 min.
14. Repeat steps 12–13 twice to wash the bead-bound CD8+ T cells. These steps are critical to obtain a high purity of isolated cells.
15. After removing the supernatant from the last wash, immediately lyse the cells with 750-1 000 ul of Trizol (with the beads, no need to detach them).

For isolation of memory subsets, we stained PBMCs with the mix of antibodies: anti-CD3-FITC (UCHT1, eBioscience), anti-CD45RA-eFluor450 (HI100, eBioscience), anti-CCR7-AlexaFluor647 (3D12, BD Pharmingen), anti-CD95-PE (DX2, eBioscience). Four subsets of cells were sorted into RLT buffer (Qiagen) on BD FACS Aria III: EM (CD3+CD45RA-CCR7-), EMRA (CD3+CD45RA+CCR7-), CM (CD3+CD45RA-CCR7+), Tscm (CD3+CD45RA+CCR7+CD95+).

1. Resuspend PBMCs in 100 ul FACS buffer (PBS 0.5% BSA 2mM EDTA) in 15 mL conical tube (alternatively FACS tubes can be used).
2. Use ~10ul to prepare single color and unstained controls.
3. Add antibodies to the cells:

anti-CD3-FITC (UCHT1, eBioscience)	5 ul
anti-CD45RA-eFluor450 (HI100, eBioscience)	5 ul
anti-CCR7-AlexaFluor647 (3D12, BD Pharmingen)	5 ul
anti-CD95-PE (DX2, eBioscience)	5 ul

For single stain controls add 1ul of antibody.

Incubate 20 minutes on ice in the dark.

1. Wash cells twice with 2 mL cold FACS buffer. Centrifuge 5 minutes, 450 g, 4C.
2. Resuspend in 300 ul FACS buffer and transfer to FACS tubes.
3. Collect cells into 100 ul RLT buffer (alternatively if more than 50 K cells are expected for the population, cells can be collected in RPMI and spin down after the sorting).

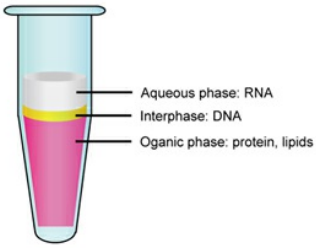
HLA-A02 dextramer loaded with the NS4B241-222 peptide (LLWNGPMAV) from YFV17D (Immudex) was used for epitope-specific T-cells isolation. Cells were stained with NS4B-dextramer-PE, anti-CD3-eFluor450 (UCHT1, eBioscience), and anti-CD8-FITC (SK1, eBioscience) according to the manufacturer's protocol.

1. For dextramer staining cells were resuspended in 90 ul FACS buffer (PBS 0.5% BSA 2mM EDTA). 5 ul was used for single color and unstained controls.
2. Add 10 ul of NS4B241-222 peptide (LLWNGPMAV) from YFV17D (Immudex) labeled with PE to the cells. Incubate 30 minutes in the dark at room temperature.
3. Add 5 ul of anti-CD3-eFluor450 (UCHT1, eBioscience), and 5 ul anti-CD8-FITC (SK1, eBioscience) and incubate for additional 20 minutes on ice.
4. Prepare single color controls with 1 ul of antibody.
5. Wash cells twice with 2 mL cold FACS buffer. Centrifuge 5 minutes, 450 g, 4C.
6. Resuspend in 300 ul FACS buffer and transfer to FACS tubes.
7. Collect cells into 100 ul RLT buffer (alternatively if more than 50 K cells are expected for the population, cells can be collected in RPMI and spin down after the sorting).

RNA was isolated using standard TriZol protocol (for bulk PBMCs, CD4 and CD8, NS4B-specific and negative fractions) or RNAeasy Micro Kit (Qiagen) (for memory subsets https://www.qiagen.com/us/resources/resourcedetail?id=5a5aebb5-1603-4a71-82f5-3eb56054417e&lang=en).
Trizol protocol

1. If cells were frozen: Defrost the cells in trizol -> 5 minutes room temp
2. For freshly collected cells: add the trizol to cells, mix well by pipetting and incubate 5-10 minutes before proceeding to the next step. If only one sample is available: proceed with 75% of trizol volume (ex from 1ml of cells + trizol mixture, we take 750 mlk) and leave 25% for later. So if something goes wrong - no RNA, drop of the tube, etc- we have at least some material to make the sample

2. Add 1/5V chloroform per 1V of trizol (0,2 ml per 1 ml), **shake in hands (hold the lid!)** for 1 minute, incubate for 3 minutes (shake several times during incubation, it should look like milk a bit)
3. Centrifuge 12000 rcf **4°C** 15 min.
4. Emulsion should be now divided in 3 phases (aqueous=RNA, interphase=DNA, Trizol).



Transfer the aqueous phase containing the RNA to a new tube (do it carefully with 200 mkl tips and not the biggest one. live some aqueous phase-several millimeters above the interphase- because if you go too close to the interphase you can catch some DNA.)
*Store the tube with the leftovers of trizol and interphase, because you can then isolate DNA if you need it later for some application.
5. Add 0.7 mkl of Pellet paint-69049 to aqueous phase and mix well by pipetting (do it before adding isopropanol)
6. Add 1/2V iPrOH per 1V of trizol (0,5ml per 1ml), mix it by rotating the tube or pipetting, incubate at room temperature for 10 minutes (RNA should precipitate after centrifugation)
7. Centrifuge max rcf 4°C 10 minutes (should see a gel-like rose pellet after it. At most cases it doesn't stick really good to the tube, so be careful not to throw it away).
8. Discard the supernatant with a pipette, add 1 ml 80% EtOH per 1ml of trizol (I prefer the freshly mixed one, like least than a week old).
STOP POINT
9. Centrifuge max rcf 4°C 10 minutes. Do the washing step again with 700 mkl of 80% EtOH.
10. Dry the pellet 45°C 5-10 minutes (open tube should be placed under the foil or some kind of a cap, we use a lid from the tip box)
11. Resuspend the pellet in 20/15/10 mkl of RNAse-free water (depending on number of cells), incubate 55°C 5 min., mix well by pipetting, do not forget to wash the sides of the tube, note that RNA itself is mostly invisible .
Store at -70, if you did it clean, it should be fine.
The amount of RNA was measured on Qubit 2.0 (Invitrogen). Information about all antibodies and commercial kits could be found in Key Resources Table.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Minervina, A. and Pogorelyy, M. (2021). Isolation of T-cell subpopulations. Bio-protocol Preprint. bio-protocol.org/prep940.
2. Minervina, A. A., Pogorelyy, M. V., Komech, E. A., Karnaukhov, V. K., Bacher, P., Rosati, E., Franke, A., Chudakov, D. M., Mamedov, I. Z., Lebedev, Y. B., Mora, T. and Walczak, A. M.(2020). Primary and secondary anti-viral response captured by the dynamics and phenotype of individual T cell clones. eLIFE. DOI: 10.7554/eLife.53704